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Subgenomic mRNA of Aura Alphavirus Is Packaged into Virions

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Purified virions of Aura virus, a South American alphavirus related to Sindbis virus, were found to contain two RNA species, one of 12 kb and the other of 4.2 kb. Northern (RNA) blot analysis, primer extension analysis, and limited sequencing showed that the 12-kb RNA was the viral genomic RNA, whereas the 4.2-kb RNA present in virus preparations was identical to the 26S subgenomic RNA present in infected cells. The subgenomic RNA is the messenger for translation of the viral structural proteins, and its synthesis is absolutely required for replication of the virus. Although 26S RNA is present in the cytosol of all cells infected by alphaviruses, this is the first report of incorporation of the subgenomic RNA into alphavirus particles. Packaging of the Aura virus subgenomic mRNA occurred following infection of mosquito (*Aedes albopictus* C6/36), hamster (BHK-21), or monkey (Vero) cells. Quantitation of the amounts of genomic and subgenomic RNA both in virions and in infected cells showed that the ratio of genomic to subgenomic RNA was 3- to 10-fold higher in Aura virions than in infected cells. Thus, although the subgenomic RNA is packaged efficiently, the genomic RNA has a selective advantage during packaging. In contrast, in parallel experiments with Sindbis virus, packaging of subgenomic RNA was not detectable. We also found that subgenomic RNA was present in about threefold-greater amounts relative to genomic RNA in cells infected by Aura virus than in cells infected by Sindbis virus. Packaging of the Aura virus subgenomic RNA, but not those of other alphaviruses, suggests that Aura virus 26S RNA contains a packaging signal for incorporation into virions. The importance of the packaging of this RNA into virions in the natural history of the virus remains to be determined.

Aura virus is an alphavirus first isolated from a pool of *Culex* (*Melanoconion*) sp. collected at ground level near Belém, Brazil, in 1959 by using human bait and was named for a small river in the region (5). It was subsequently isolated from two different pools of *Aedes serratus* collected in the same area over the next 2 years. Serological surveys of residents of the area revealed that only 1 of 1,313 human sera had antibodies that inhibited hemagglutination by Aura virus and that these antibodies were of low titer, and there is no evidence for human disease caused by the virus. Only one serum from 288 marsupials, one from 955 rodents, and one from 48 horses, and none from monkeys, edentates, bats, lizards, rabbits, carnivores, birds, frogs, cows, and sheep, contained antibodies that inhibited hemagglutination by Aura virus, and the primary vertebrate host of the virus is unknown (5). Aura virus was later isolated from *A. serratus* collected in Missiones Province, Argentina, in 1966, and the virus thus appears to have a wide geographic distribution (2, 3). Serological studies of Aura virus showed that it was an alphavirus belonging to the Sindbis virus/western equine encephalitis (WEE) virus complex but was distinct from both WEE virus and Sindbis virus (4). We reported recently that WEE virus is a recombinant virus; most of its genome is derived from eastern equine encephalitis (EEE) virus, and only the two viral glycoprotein genes are derived from a Sindbis-like virus (9). The antigenic classification of WEE virus as a member of the Sindbis virus subgroup is thus a function only of the envelope glycoproteins. Because WEE and EEE viruses are found only in the Americas whereas Sindbis virus has been reported only in the Old World, we have begun to investigate South American viruses belonging to the WEE/Sindbis virus complex in order to clarify the status of Sindbis-like alphaviruses in the New World.

The plus-stranded RNA genome of alphaviruses is about 11.8 kb in size, including a 3′ poly(A) tract, and is divided into a 5′ region encoding the nonstructural proteins required for RNA replication and a 3′ region encoding the structural proteins that are assembled with the genomic RNA into progeny virions (23). The structural proteins are translated from a 4.2-kb subgenomic RNA transcribed from a full-length minus-strand template during virus replication. The promoter for subgenomic RNA transcription is well understood (10, 16, 18), and in infected cells, this RNA is present in molar excess over the genomic RNA. Despite this excess, however, the subgenomic RNA is not packaged into virions in the case of any alphavirus studied heretofore. Recent studies of defective interfering (DI) RNAs of Sindbis virus have shown that there is a packaging signal in the genomic RNA that is required for efficient encapsidation of RNA in the infected cell (26). This signal is present in a region of the genome that is not transcribed into subgenomic RNA, which could explain the exclusion of the subgenomic RNA from progeny virions. In this paper, we report that in the case of Aura virus, subgenomic RNA is efficiently packaged into virions, and we conclude that a packaging signal for Aura virus RNA lies in the region of the genome that is transcribed into subgenomic RNA.

MATERIALS AND METHODS

Cells and virus. Aura virus, prototype strain BeAR 10315, was obtained from Joel Dalrymple, U.S. Army Medical Research Institute of Infectious Diseases, and from the American Type Culture Collection and represented the ninth suckling mouse brain passage. These stocks were found to behave identically. Infectious virus was handled under BL-3 contain-

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ment. Sindbis virus was our laboratory HRSP strain derived from the prototype AR339 strain.

BHK-21 clone 15 cells, Vero cells, *Aedes albopictus* C6/36 cells, and primary chicken embryo fibroblasts were grown in Dulbecco modified Eagle medium supplemented with 10% (BHK, Vero, and C6/36 cells) or 2% (chicken cells) fetal calf serum at 30°C (mosquito cells) or 37°C (vertebrate cells) in 5% CO_2. Virus titers were determined by plaque assay on BHK-21 cells. The growth of Aura virus in the different cells was assayed by infecting cell monolayers at a multiplicity of 1 for 1 h followed by removal of the inoculum, washing of the cell monolayer, and addition of culture medium. At selected time intervals, the medium was removed and replaced with fresh medium; virus released into the medium was assayed by plaque assay.

Preparation of RNA and hybridization experiments. Virus-containing culture fluid was collected 18 to 32 h after infection, and the virus was pelleted by centrifugation at 23,000 rpm for 4 h at 4°C in a Beckman SW27 rotor. The virus pellet was resuspended in TNE buffer (50 mM Tris-Cl [pH 7.4], 200 mM NaCl, 5 mM EDTA), and the virus was fractionated by centrifugation in a sucrose gradient (10 to 30% sucrose in TNE) at 32,000 rpm for 1.5 h at 4°C in a Beckman SW41 rotor. The visible bands of virus were removed by syringe and diluted 10-fold with TNE, and the virus was pelleted by centrifugation at 35,000 rpm for 1 h at 4°C in a Beckman SW50 rotor. The virus pellet was resuspended in 50 mM Tris-Cl (pH 7.5) containing 10 mM EDTA and 0.5% sodium dodecyl sulfate (SDS), the solution was extracted twice with phenol (equilibrated to pH 8.0) and chloroform, and the RNA was precipitated with ethanol after addition of sodium acetate (pH 5.3) to 0.3 M.

Aura virus RNA (2 to 5 μg) was treated with 2 M glyoxal at 56°C for 30 min and subjected to electrophoresis in a denaturing formaldehyde-agarose gel (15% formaldehyde solution, 1% agarose) in 10 mM Na_2HPO_4 buffer (pH 6.8) with buffer circulation. RNA was stained with acridine orange (33 μg/ml) in running buffer for 5 min and then destained for 2 h in the same buffer, using five buffer changes. After capillary blotting to a nylon membrane, hybridization was performed with cDNA derived from Sindbis virus (clone Toto54 from R. Kuhn). The 7.6-kb *Sac*I-*Mlu*I fragment was used as a probe for the nonstructural protein region of the genomic RNA, and the *Mlu*I-*Xho*I fragment was used as a probe for the structural protein region. Hybridization at 48°C in 500 mM Na_2HPO_4 (pH 7.2) containing 7% SDS was followed by washing at the same temperature with 50 mM Na_2HPO_4 (pH 7.2) containing first 5% SDS and then 1% SDS.

RNA from infected cells was prepared by the guanidium isothiocyanate method of Chirgwin et al. (6) followed by centrifugation through 5.7 M CsCl.

cDNA cloning. For cDNA synthesis, 3 μg of Aura virus RNA from purified virions was annealed to 100 pmol of oligo(dT) (Pharmacia) and incubated with 20 U of avian myeloblastosis virus reverse transcriptase in 50 mM Tris (pH 8.35)–50 mM KCl–8 mM $MgCl_2$–2.5 mM dithiothreitol at 42°C for 1 h. Second-strand synthesis was initiated by incubation with 1 U of RNase H, 2 U of DNA polymerase I, and 1 U of *Escherichia coli* DNA ligase in 0.1 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 6.9)–7 mM KCl–4 mM $MgCl_2$–1.5 mM β-mercaptoethanol–1.5 mM β-NAD at 37°C for 1 h. The cDNA was ligated to *Eco*RI adapters (Pharmacia), size selected with a lower cutoff of 4 kb, phosphorylated, and cloned into *Eco*RI-digested lambda ZapII (Stratagene). The resulting library yielded 2×10^5 individual clones and was amplified once. Clones containing Aura virus inserts were identified by using a nick-translated probe which included nucleotides 56 to 8330 of Sindbis virus. Sequences determined from the clones with the longest inserts were used to generate the primer oligonucleotide Au1 (5′ GCCAAACGGATTGT AA 3′), which was used for primer extension and as a primer for the generation of a second cDNA library covering the nonstructural genes of Aura virus RNA. Clones from this library served for the determination of the 26S promoter sequence.

Primer extension analysis. Two micrograms of RNA from purified Aura virus propagated on BHK cells or on mosquito C6/36 cells, or 10 μg of RNA from Aura virus-infected BHK cells, was heated to 65°C for 5 min and annealed to ^{32}P-labeled oligonucleotide primer Au1 in 1× reverse transcriptase buffer. Deoxynucleoside triphosphates (0.5 mM) and 10 U of avian myeloblastosis virus reverse transcriptase were added, and the mixture incubated at 42°C for 15 min and then heated to 95°C for 5 min in 50% formamide. The extension product was analyzed on a 6% sequencing gel.

Quantitation of viral RNAs. Cells (10^7) were infected with Aura virus at a multiplicity of 1 and labeled with 200 μCi of [^{3}H]uridine in the presence of 10 μg of dactinomycin per μl between 12 and 24 h after infection. RNA from virus or infected cells was prepared as described above and subjected to electrophoresis on native or denaturing agarose gels. The 26S and 49S RNA bands were located by staining with acridine orange or by autoradiography, the bands were excised from the dried gel, and the radioactivity was quantitated by liquid scintillation counting after solubilization in Readysolv (NEN).

RESULTS

Growth of Aura virus. The growth of Aura virus was examined on four cell lines, mosquito (C6/36), chicken (primary embryo fibroblasts), hamster (BHK-21), and monkey (Vero). The results of an experiment in which the medium was changed at 4, 6, 8, 12, 24, 36, and 48 h are shown in Fig. 1. BHK and C6/36 cells produced large amounts of virus. Yields of infectious virus by these cells were about 10^6 PFU/ml/h between 8 and 36 h for BHK cells or 12 and 48 h for mosquito cells (Fig. 1). The specific infectivity of Aura virus grown in BHK cells was estimated from the amount of RNA isolated from virus preparations (see below). We found that there were about 10^3 RNA-containing particles per PFU, and thus BHK cells, and presumably mosquito cells, produce about 10^9 particles per ml per h, or 3×10^{10} particles per ml during 30 h of virus production. These yields of particles are comparable to those produced by other alphaviruses such as Sindbis virus, but in Sindbis virus, the particle/PFU ratio is much lower, on the order of 2 to 10 (21). Vero cells produced much less virus, about 10^4 PFU/ml/h. Chicken cells were essentially nonpermissive for Aura virus growth, producing $<10^3$ PFU/ml/h. The infection cycle of Aura virus in vertebrate cells is protracted in comparison with that of Sindbis virus; in the latter case, virus production ceases by 12 to 24 h after infection because the infected cells disintegrate.

Presence of 4.2-kb RNA in virus particles. Aura virus was grown in BHK cells, virions were purified, and RNA was prepared from the purified virions. Two sizes of RNA, 12 and 4.2 kb, were observed after electrophoresis of the RNA on a denaturing agarose gel, as shown in Fig. 2A. The 4.2-kb band and 12-kb band were of approximately equal intensity in the stained gel, indicating that the 4.2-kb RNA is present in a threefold molar excess over the 12-kb RNA. As a control, RNA from purified Sindbis virions is also shown on this gel, and there is no 4.2 kb RNA detectable.

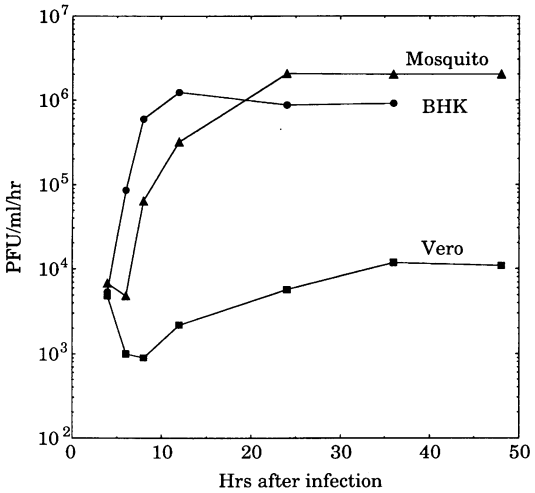

FIG. 1. Growth of Aura virus in different cells. Monolayers containing 1×10^7 to 2×10^7 BHK, Vero, or mosquito C6/36 cells were infected with Aura virus at a multiplicity of 1 for 1 h. The inoculum was removed and replaced with 10 ml of medium, and the medium was changed at 4, 6, 8, 12, 24, 36, and 48 h (except in the case of BHK cells, where the monolayer had disintegrated by 36 h). Titers of Aura virus released into the medium during each period were determined by plaque assay on BHK cells. The titers were divided by the number of hours of incubation for each sample, and PFU released per milliliter per hour was plotted. Chicken embryo fibroblasts were also infected in the same experiment; the titers obtained were $<10^3$ PFU/ml/h.

The 12-kb RNA is slightly larger than the genomic RNA of Sindbis virus (Fig. 2A) and must represent the Aura virus genome, but the identity of the 4.2-kb RNA was not immediately clear. We first examined the possibility that the 4.2-kb RNA arose from DI particles, the presence of which could explain the low titer of Aura virus produced (20). Two rounds of four sequential plaque purifications, which is normally

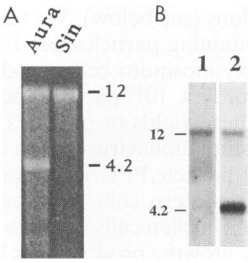

FIG. 2. Examination of RNA from Aura and Sindbis viruses. (A) Aura virus and Sindbis virus were propagated in BHK cells, and virus was purified by velocity sedimentation on sucrose gradients. RNA was isolated from purified virions, and 2 μg of RNA was treated with glyoxal and subjected to electrophoresis in a denaturing agarose gel. The gel was stained with acridine orange before photography. The sizes of the two Aura virus bands are indicated on the right in kilobases. (B) Aura virus RNA was isolated from purified virions, and 2 μg was treated with glyoxal and subjected to electrophoresis on a denaturing agarose gel. RNA was transferred to a nylon membrane and probed with one of two probes derived from a cDNA clone of Sindbis virus. The probe in lane 1 was ^{32}P-labeled cDNA containing only the nonstructural region of the Sindbis genome (nucleotides 1 to 7600), and the probe in lane 2 contained only the structural region of the genome (nucleotides 7600 to 11700).

effective in removing DI particles from an alphavirus population, did not alter the amount of 4.2-kb RNA present in purified virions. In a second approach, we used plaque purification following transfection of cells with total Aura virus RNA isolated from virions, and this also had no effect on the presence of the 4.2-kb RNA.

Identification of the 4.2-kb RNA. The 4.2-kb RNA is of the exact size expected for the subgenomic 26S mRNA of Aura virus, and we next examined the possibility that it represents packaged subgenomic RNA. RNA from purified Aura virions was treated with glyoxal, separated on a denaturing agarose gel, and transferred to a nylon membrane. The membrane was then probed with cDNA from Sindbis virus. A cDNA probe containing the entire 7.6-kb nonstructural region but none of the structural region reacted exclusively with the 12-kb Aura virus RNA (Fig. 2B, lane 1), whereas a probe containing only the 4.1-kb structural region reacted with both Aura virus RNAs (Fig. 2B, lane 2). These results are consistent with the hypothesis that the 12-kb RNA is the Aura virus genome and the 4.2-kb RNA is the subgenomic 26S RNA. The cross-hybridization observed also shows that Aura and Sindbis viruses are closely related, consistent with the close antigenic relationships observed (4) and with sequence data for Aura virus (19).

We next prepared a cDNA library of Aura virus RNA from purified virions, using oligo(dT) priming, and sequenced the ends of several clones with ~4-kb inserts, which were expected to arise primarily from the 4.2-kb RNA. The sequence was aligned to that of Sindbis virus, and the results indicated that the 4-kb clones were derived from Aura virus 26S RNA. From this sequence, we synthesized an oligonucleotide that binds to Aura virus RNA 55 nucleotides downstream of the presumed 5′ end of Aura virus 26S RNA and used it for primer extension with RNA from purified virus as well as RNA from infected cells. The extended primer terminated at the identical point with RNA isolated from viruses as with RNA from cells (Fig. 3). This termination point is precisely where 26S RNA is predicted to begin from sequence homology with Sindbis virus. Thus, it is clear that the ends of the 4.2-kb RNA packaged into Aura virions are identical to those of the subgenomic RNA produced in infected cells. From this finding, the size of the RNA, and the hybridization results in Fig. 2B, we conclude that the 4.2-kb RNA is subgenomic mRNA that is packaged in the case of Aura virus but not in the case of Sindbis virus.

In Fig. 4, the sequences of the 5′ and 3′ ends of 26S Aura virus RNA derived from sequencing of cDNA clones and from primer extension are shown aligned with the sequence of Sindbis virus. The close relationship between Aura and Sindbis viruses is evident.

Distribution of 26S RNA. To determine the ratio of genomic to subgenomic RNA in infected cells and in virions, infected cells were labeled with [^{3}H]uridine in the presence of dactinomycin, and RNA from cells or from purified virions was

TABLE 1. Relative concentrations of RNA species after alphavirus infection of different tissue culture cell lines

Prepn	Molar ratio, 26S/49S			
	Aura virus			Sindbis virus, BHK-21
	BHK-21	Vero	C6/36	
Cytosol	8.6	9.0	11.0	3.4
Virus	3.3	2.1	1.1	—[a]

[a] No 26S RNA could be detected in Sindbis virions (see Fig. 2A).

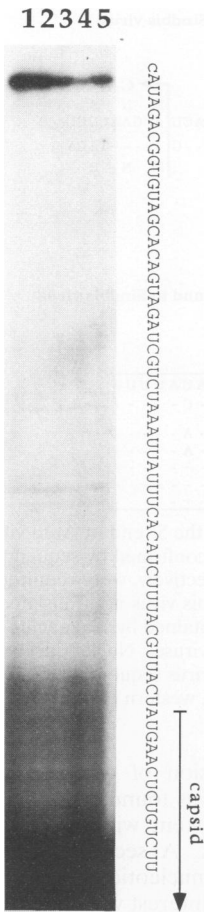

FIG. 3. Analysis of the 5′ end of Aura virus subgenomic RNA by primer extension. BHK cells (lanes 1 to 3) or mosquito C6/36 cells (lanes 4 and 5) were infected with Aura virus, and RNA was prepared from purified virus (lanes 2 to 4) or from infected cells (lanes 1 and 5). A synthetic primer that binds to the Aura virus sequence 55 nucleotides from the expected start of the subgenomic RNA was used for primer extension, and the products are displayed on a sequencing gel. Note that all products terminate at the same position. The sequence of the 5′ end of the subgenomic RNA (derived from dideoxy sequencing experiments using the same primer and from cDNA sequence) is shown on the right. The start of the sequence encoding the capsid protein is also indicated.

examined by electrophoresis in denaturing agarose gels. The amount of label in 49S and 26S RNAs was quantitated by liquid scintillation counting; the results, expressed as a molar ratio relative to 49S RNA, are shown in Table 1 for three different cell lines. In all three cell lines, the molar ratio of 26S to 49S RNA in the infected cell was approximately the same, with 26S RNA being present at 9 to 11 times the molar amount of 49S RNA. In contrast, 26S RNA was produced in lesser relative amounts in Sindbis virus-infected cells, being present at 3.4 times the level of 49S RNA in infected BHK cells (Table 1).

The molar ratio of 26S RNA to 49S RNA in Aura virus particles (1.1 to 3.3) was less than the molar ratio of these two RNAs within the cell (9 to 11). The ratio in virus particles was dependent on the cell in which the virus was grown, being 3.3 for BHK-grown virus, 2.1 for Vero-grown virus, and 1.1 for C6/36-grown virus (Table 1). Thus, packaging of 26S RNA is efficient, especially in BHK cells, but 49S RNA is packaged 3- to 10-fold more efficiently than 26S RNA, depending on the cell infected. In contrast, no 26S RNA could be detected in purified virions of Sindbis virus (Fig. 2A).

In the case of BHK-grown virus, the number of 26S RNA-containing virions is greater than the number of 49S RNA-containing virions, even if there are three subgenomic RNAs packaged per particle. This may in part explain the low yield of infectious particles from Aura virus-infected cells. However, other factors must also be involved because the specific infectivity of 49S RNA-containing particles is only ~0.002, much less than the specific infectivity found for other alphaviruses.

The subgenomic promoter. The increased production of 26S RNA relative to 49S RNA in Aura virus-infected cells in comparison with its very close relative Sindbis virus led us to examine the subgenomic promoter (16, 18). The Aura virus-specific primer that primes just downstream of the start of 26S RNA was used to prime cDNA synthesis on viral RNA, and cDNA clones were isolated. Clones with inserts greater than 70 nucleotides in length must arise by priming on genomic RNA rather than subgenomic RNA. Sequence of the ends of selected clones provided the sequence of the Aura virus junction region and thus of the subgenomic promoter. This sequence of the minimal promoter (16, 18) is shown in Fig. 5 and compared with the sequence of the Sindbis virus subgenomic promoter and with the sequence for several other New World alphaviruses. The Aura virus promoter is almost identical to that of Sindbis virus. There are only two differences from the Sindbis virus sequence, both of which are located near the very ends of the promoter in nucleotides that are variable among New World alphaviruses. The Aura virus promoter is less similar to those of other New World alphaviruses. It appears unlikely that the two changes between the Sindbis virus and Aura virus promoters account for the difference in transcription efficiency of 26S RNA, but rather that differences in the sequence upstream of the promoter, which is known to enhance the activity of the promoter (18), or changes in the virus nonstructural proteins are responsible.

DISCUSSION

Wengler et al. (27, 28) reported that the Sindbis virus capsid protein would assemble into core-like particles in vitro in the presence of any RNA, including Sindbis virus 49S or 26S RNA, tRNA, or even polyanionic polymers. However, several different studies have suggested that there are specific encapsidation signals in alphavirus RNA required for efficient formation of nucleocapsids in infected cells. First, for most alphaviruses, only the genomic 49S RNA is (detectably) encapsidated despite the molar excess of 26S subgenomic RNA in the cell, as shown in Fig. 2A and in numerous earlier studies, suggesting the presence of a packaging signal in the genomic RNA. Second, in a recent study with Semliki Forest (SF) virus, overexpression of capsid protein in cells in the absence of genomic RNA led to the formation of only very small amounts of nuclecapsids (24), also suggesting that an encapsidation signal in the genomic RNA is required for efficient packaging of the RNA. Third, DI RNAs appear to require packaging signals that are distinct from replication promoters, since DI RNAs that replicate well but are packaged poorly have been observed (11). Finally, recent work of Weiss et al. (26) showed directly that at least one copy of the sequence between nucleotides 948 and 1226 of Sindbis virus RNA (which is found in the nonstructural region and thus is not present in 26S RNA) was required for efficient packaging of a Sindbis virus

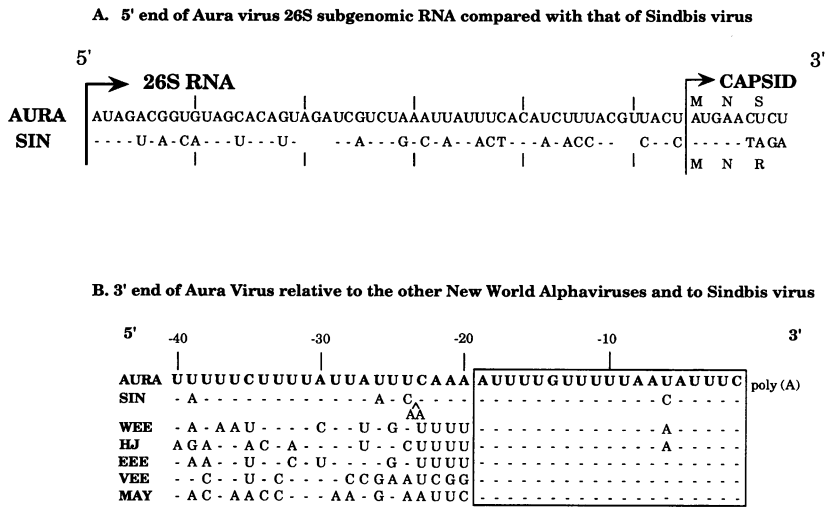

FIG. 4. Sequences of the 5′ and 3′ ends of Aura virus subgenomic RNA. (A) The sequence of the 5′ end of Aura virus subgenomic RNA was determined by direct dideoxy sequencing of subgenomic RNA derived from purified virions and confirmed by sequencing of cDNA clones. The sequence is aligned with that of Sindbis (SIN) virus. Two gaps of three and two nucleotides, respectively, were required in the Sindbis sequence for alignment, as the Aura virus 5′ nontranslated region is five nucleotides longer than the Sindbis virus nontranslated region. The start of the capsid protein sequence is also indicated. (B) The sequence of the 3′ end of Aura virus RNA was obtained by sequencing the ends of cDNA clones, and the sequence is shown aligned with that of Sindbis virus and of several other New World alphaviruses. Nucleotides are numbered 3′ to 5′ from the poly(A) tract. The 19-nucleotide 3′-terminal conserved sequence element is boxed. The Aura virus sequence is more similar to that of Sindbis virus than it is to that of other New World alphaviruses. Virus abbreviations: SIN, Sindbis; WEE, western equine encephalitis; HJ, Highlands J; EEE, eastern equine encephalitis; VEE, Venezuelan equine encephalitis; MAY, Mayaro.

DI RNA, and this sequence is thus operationally a packaging signal or a part of a packaging signal. These authors also found that the RNA sequence from nucleotide 746 to 1226 bound to capsid protein in an in vitro binding assay and that the 948–1226 region also bound to capsid protein but less strongly. Deletion of the 5′ part of the longer domain was found to reduce the replication efficiency of the DI RNA, and this 5′ region appears to promote RNA replication instead of, or in addition to, encapsidation. Further studies are required to determine whether the 948–1226 region represents the complete encapsidation signal of Sindbis virus or whether the longer domain from nucleotide 746 to 1226, or other regions of the genome as yet undefined, form part of the encapsidation signal.

The Sindbis virus sequence from nucleotides 948 to 1226 is found twice in a naturally occurring DI RNA (17). The presence of multiple copies of a packaging signal may increase the efficiency of encapsidation. In one study with SF virus, it was found that when DI RNAs first arose they were packaged poorly, but with continued passage, the DI RNAs were packaged more efficiently (11). One possible interpretation of this result is that with passage, multiple copies of the packaging signal become established in the DI RNA.

Two DI RNAs from SF virus have been sequenced, and examination of these sequences suggests that a packaging signal in this virus is found between nucleotides 2737 and 2993, also in the nonstructural region but distinct from the placement of the Sindbis virus signal. One DI RNA sequenced by Lehtovaara et al. (12) had a complexity of only 680 nucleotides (but a total length of 1,652 nucleotides); nucleotides 11 to 317, nucleotides 2728 to 2994, and the 3′ 106 nucleotides of the SF virus genome were the only sequences present in the DI RNA, and any encapsidation signal required to package SF virus DI RNA must be contained within these sequences. Furthermore, nucleotides 35 to 317, 2729 to 2857, and 2918 to 2993 were each present in three copies. Although these sequences have not been directly shown to be required for encapsidation of SF virus RNA, expression of the DI RNA sequences in SF virus-infected cells was found to inhibit formation of SF virus nucleocapsids, consistent with the presence of a packaging signal in this RNA. A second DI sequenced (13) had a complexity of 1,169 nucleotides (total length of 2,286 nucleotides); of particular interest was the fact that nucleotides 2737 to 3010 were present in four copies. Three additional SF virus DI RNAs have been examined by electron microscopic heteroduplex analysis (1). Although this method is less precise than sequencing, the conclusion was reached that these three RNAs had three regions in common with one another and with the two sequenced DI RNAs. These were the 3′-terminal 80 nucleotides, the 100 nucleotides between nucleotides 200 and 300, and 90 nucleotides near position 2740. Taken together, these results suggest that an SF encapsidation signal is found between nucleotides 2737 and 2993. It is of particular interest that the capsid binding domain between nucleotides 746 and 1226 of Sindbis virus is absent from SF virus DI RNAs, and the 2737–2993 domain of SF virus found in multiple copies in DI

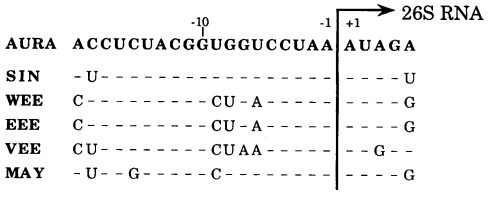

FIG. 5. Sequence of the Aura virus subgenomic promoter. The sequence of the Aura virus subgenomic promoter was obtained by sequencing the ends of cDNA clones obtained by using a primer that binds Aura virus RNA 55 nucleotides downstream of the start of 26S RNA. This sequence is compared with that of the Sindbis virus promoter and with those of the promoters of several New World alphaviruses. The start of 26S RNA is indicated. Abbreviations are as in the legend to Fig. 4.

RNAs is absent from Sindbis virus DI RNAs. Although a priori it would have seemed likely that two alphaviruses would have a related encapsidation signal, these results strongly suggest that the position of the encapsidation signal is different in Sindbis and SF viruses.

Our results with Aura virus strongly support the hypothesis that alphavirus RNAs contain one or more packaging signals required for encapsidation of the viral RNA in infected cells. In contrast to the case for Sindbis virus or SF virus, the Aura virus subgenomic RNA is packaged into virions. The yields of Aura virus particles are comparable to those produced following Sindbis virus infection, and it seems unlikely that the presence of Aura virus 26S RNA in virions results from efficient encapsidation of any RNA by the Aura virus capsid protein, i.e., that no packaging signal is required for efficient packaging of Aura virus RNA even though a packaging signal is required for efficient packaging of other alphavirus RNAs. The results with Aura virus also rule out the possibility that the failure of alphaviruses to package 26S RNA arises from compartmentalization within the infected cells such that the 26S RNA is unavailable for packaging and indicate that the failure of other alphaviruses to package 26S RNA derives from the absence of a packaging signal in their 26S RNAs. Our results imply that there is an encapsidation signal in Aura virus RNA within the region transcribed into the subgenomic mRNA, a domain comprising approximately nucleotides 7700 to 11700 of the alphavirus genome, a position very different from that for the putative Sindbis virus or SF virus signals. It remains to be determined whether this Aura virus encapsidation signal represents the major or only encapsidation signal in Aura virus RNA or whether there is another, presumably related, signal in the nonstructural region. Complete sequencing of Aura virus RNA, study of DI RNAs, and study of deletion viruses derived from full-length cDNA clones would be of great interest in unraveling these features. Whether encapsidation of 26S RNA is important for the natural history of Aura virus also remains to be determined.

Aura virus 26S RNA is packaged less efficiently than the genomic 49S RNA, as demonstrated by the difference in 26S/49S RNA ratios in virions and in the cell. This could be due in part to sequestration of 26S RNA by ribosomes but for the most part seems likely to be due to a size requirement of the RNA for efficient encapsidation. DI RNAs are packaged less efficiently than are full-length genomes (11), despite the fact that these RNAs appear to contain multiple copies of an encapsidation signal and do not serve as mRNA. It seems quite likely that DI RNAs are selected to contain multiple encapsidation signals in order to compete more effectively with the genomic RNA during packaging. Even though the less efficient packaging of 26S RNA probably arises primarily from its smaller size, the apparent dependence of the extent of 26S RNA packaging upon the host cell suggests that host factors might also be involved. Alternatively, 26S RNA could be sequestered to different extents in these different cells, such as by more efficient translation in mosquito cells leading to a larger fraction of RNA being present in polysomes.

The elevation in 26S RNA levels relative to 49S RNA following Aura virus infection, compared with its close relative Sindbis virus, could be due to a more potent promoter for subgenomic RNA transcription or to an increased preference of the Aura virus RNA transcriptase for subgenomic RNA synthesis. Current models for control of 26S RNA transcription suggest that alternative processing pathways for polyprotein precursors are involved in regulation of genomic, subgenomic, and antigenomic RNA synthesis (7–9, 14, 15, 22). The production of subgenomic RNA is probably regulated so that an optimum amount of structural proteins is produced, and very high levels of capsid protein in cells may be toxic (25).

Finally, the low specific infectivity of Aura virus is of interest. It is possible that assembly of the virus is not regulated properly, perhaps because of interference by 26S RNA, and the virus particles misassemble or inactivate rapidly. A second possibility is that the virus is very dependent on host cell functions for replication, and the infection cycle may have a high probability of aborting because the cells studied are only semipermissive for Aura virus replication.

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